Chapter 3

Glycosylation of Integrins in Melanoma Progression

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Additional information is available at the end of the chapter

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Abstract

Each stage of melanoma development from transformed melanocytes to metastatic lesions requires the involvement of cell adhesion receptors, among which integrins are of particular importance. Strong N-glycosylation of αβ integrin heterodimers influences their processing, activation, and functions related to the modulation of cell adhesion to extracellular matrix proteins (ECM) and the basement membrane. A lack of N-glycans on integrin chains significantly reduces their interactions with the ECM. Melanoma progression is accompanied by changes in the composition of N-glycans on integrin subunits. The glycosylation profile of integrins depends on the stage of melanoma development and on the location of the metastasis. Enhanced expression of β1,6-branched complex-type oligosaccharides and altered sialylation are well-characterized changes in the N-glycosylation of integrins observed in melanoma progression. This chapter summarizes the current state of knowledge about α3β1, α5β1, and αvβ3 integrin glycosylation in melanoma and the functional consequences of changed glycosylation for the development of this cancer.

Keywords: integrin, N-glycosylation, melanoma, β1,6 branching, migration, extracellular matrix proteins

1. Introduction

Melanoma progression and the acquisition of invasive and metastatic competence by melanoma cells are accompanied not only by changes in integrin expression but also by alterations of the sugar component of these heavily N-glycosylated adhesive proteins [1]. This post-translational modification is critical to integrin functions, mainly its interactions with extracellular matrix proteins (ECM) and the basement membrane [2]. Changes in the expression and glycosylation of integrins contribute to each stage of melanoma progression.

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Human cutaneous melanoma develops in a series of definable stages, from the common acquired nevus and dysplastic nevus through the radial growth phase (RGP) and vertical growth phase (VGP) of primary melanoma and finally metastatic melanoma. During these multistep transformations, melanoma cells acquire the ability to invade the dermis and then disseminate throughout the body via blood and lymphatic vessels [3–8]. Adjustment of integrin glycosylation is an important feature of the melanoma cell's adaptation to the constantly changing conditions of its microenvironment. This chapter reviews the current state of knowledge about integrin glycosylation in the course of melanoma progression.

2. Overall characteristics of integrins

The term "integrins" introduced by Hynes reflects the capacity of these cell surface receptors to integrate ECM proteins with the cytoskeleton and with intracellular signaling pathways by physical connection [9]. The role of integrin-mediated adhesion to the ECM in cell survival is now accepted. Integrins are heterodimeric cell surface adhesion molecules consisting of α and β subunits. By combining 18 α with 8 β subunits, at least 24 integrin dimers can be formed, each with its own characteristic specificity for ligands [10] (**Figure 1**).

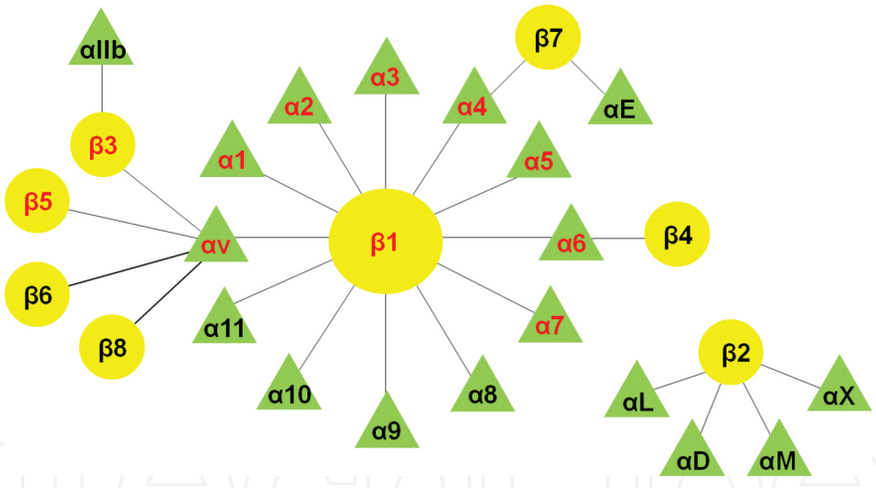

Figure 1. Integrin classification based on β subunits possessed in common. Integrin heterodimers whose expression was observed to increase during melanoma progression are marked in red.

2.1. The structure of α and β subunit ectodomains

Each integrin subunit consists of a large extracellular domain and short transmembrane and cytoplasmic domains. The extracellular domains (ectodomains) of the α and β subunits are constructed of several subdomains joined together by flexible linkers [11, 12]. The crystal structure of the αvβ3 [13] and αIIbβ3 [14] ectodomains has been characterized in detail.

The ectodomain of the α-subunit contains four or five elements: a seven-bladed β-propeller, a thigh, and two calfs. There are also nine integrins with an α-subunit containing an additional

α-I domain inserted between blades 2 and 3 of the β-propeller. A structure similar to an α-I domain is also present in the β subunit of integrins. The β-propeller contains Ca^{2+}-binding sites needed for ligand binding. The thigh and calf of the α-subunit have 140–170 residues folded into an immunoglobulin-like domain.

The ectodomain of the β-subunit consists of seven subdomains: a PSI (plexin-semaphorin-integrin), an Ig-like hybrid, a β-I-like domain, and four EGF-like modules (epidermal growth factor-like modules), followed by the β-tail part. The β-I-like domain is inserted into the hybrid modules and shows homology to the α-I domain. The PSI domain is split into two parts. The α-I domain is the primary region of ligand binding in integrins that have this structure, whereas the other integrins form the binding site through the cooperation of both subunit ectodomains (β-propeller/β-I-like interface) [15]. It has been suggested that the I-domain can exist as an "open" (high-affinity) or "closed" (low-affinity) conformation. The presence of a "metal-ion-dependent-adhesion-site" (MIDAS) motif indicates the role of divalent metal ions in achievement of the high-affinity state by integrins.

The transmembrane segments of each subunit are followed by a short cytoplasmic tail. Although they have no enzymatic activity, cytoplasmic tails play an important role in integrin activity and signal transfer.

2.2. Bidirectional signaling of integrins

Integrins are involved in bidirectional signaling—inside-out and outside-in—through their function as a linker between the ECM and the cytoskeleton [16, 17]. Control of the integrin conformation state is required for their signaling. There is little agreement among the findings from nuclear magnetic resonance (NMR) studies of cytoplasmic tails [12], but other data support the view that transmembrane and cytoplasmic domains play a key role in this signaling. In the inactive state, these domains are closely associated; separation of the chain results in activation of adhesion [11, 16].

Inside-out activation is mediated by talin binding to the β-tail, which interrupts the α/β interaction [18]. In fact, a large number of proteins have been shown to interact with cytoplasmic domains of integrins, among them cytoskeleton proteins (talin, filamin, and kindlins), adaptor proteins, and kinases [11, 19]. Talin and kindlins bound to β-integrin cooperate to regulate integrin affinity [19]. Upon binding of the ligand to the integrins' extracellular domain, signal transduction to the cytoplasm is transmitted in the classical direction: outside-in. Generation of intracellular signals leads to the formation of a focal adhesion complex which involves over 150 intracellular proteins and serves as a center of intracellular signaling [20]. Among these proteins are scaffolding molecules and also kinases such as focal adhesion kinase (FAK) and Src family kinase (SFK). So the function of integrin is related to its ligand affinity, which can be induced either by conformational changes or by clustering on the cell surface [11].

2.3. Classification of integrins and their ligands

The first classification of integrins was based on the presence of a common β subunit having distinct α subfamilies. Recent work has shown that one α subunit may associate with different

β subunits, in particular a αv subunit. However, the largest number of integrins are still assigned to the β1 (VLA, very late-activated antigens) subfamily. In this group, are integrins recognizing fibronectin (FN) (α5β1, α4β1), collagen (α1β1, α2β1, α10β1, α11β1) or laminin (LN) (α2β1, α3β1, α6β1, α7β1) [21, 22] (**Figure 1**). The α4β1 integrin present on human lymphocytes has been shown to bind vascular cell adhesion molecule 1 (VCAM-1), the cell surface protein of activated endothelia. The β2 subfamily of integrins is limited to white blood cells. Recognition of cell surface receptors of the Ig superfamily by β2 integrins is crucial to leukocyte–endothelium interaction [22, 23]. The β3 subfamily consists of two members: platelet receptor (αIIbβ3) and vitronectin - receptor (αvβ3). Integrin αIIbβ3 is specific for platelets; it recognizes fibrinogen specifically but upon platelet activation can also bind fibronectin (FN), von Willebrand's factor and thrombospondin. Integrin αvβ3 binds multiple ligands including vitronectin (VN), fibrinogen, thrombospondin, and von Willebrand's factor [23]. αv subunit can associate with more than one β subunit, such as β1, β5, β6, and β8 [22].

Integrins bind to a specific motif in their ligands. The RGD (Arg-Gly-Asp) sequence found within matrix proteins including FN, VN, thrombospondin, and laminin (LN) is usually recognized by integrins [12, 23], but there are integrins that recognize their ligands through motifs other than RGD. Integrins, α3β1, α6β1, and α7β1, being highly specific LN receptors, bind to different regions of this ligand [12]. Fibrinogen contains the binding sequence Lys-Gln-Ala-Gly-Asp-Val, while Asp-Gly-Glu-Ala was found to be the dominant binding motif in type I collagen [24].

3. Integrin expression in melanoma

Changes in integrin expression have been studied extensively in melanoma carcinogenesis [8, 25]. The integrin profile of melanoma cells differs significantly from that of normal melanocytes [26, 27] and is closely related to the stage of melanoma progression [24, 28]. Flow cytometry showed significant differences in the expression of α2, α3, β1, and especially α5 integrin subunits between WM35 primary and two metastatic human cell lines (WM9 and A375), indicating that acceleration of melanoma invasion is accompanied by increased integrin subunit synthesis [29]. Significant up-regulation of α5 integrin expression was also shown in highly metastatic B16-F10 murine melanoma cells as compared to weakly metastatic B16-F1 cells [30]. A low level of α3β1 integrin was found in benign lesions of primary melanoma, whereas in malignant cutaneous melanoma, the expression of the heterodimer progressively increased and was connected with the degree of invasion into the dermis [31].

It is well documented in *in vitro* models that melanoma development and acquisition of the metastatic phenotype are also correlated with the expression of αvβ3 integrin [26, 32, 33]. An early study by Albelda et al. [34] showed that the β3 subunit is restricted to the VGP and metastatic melanomas; in the RGP and in nevus cells, this integrin chain was not found. A study of pairs of differing melanoma cells taken from the same patient (primary WM115 and metastatic WM266-4 cell lines) supported previous observations that in primary melanoma the cells survive without αv integrins, while in disease progression, their growth and functions

depend on this receptor's expression [35]. Our group detected αvβ3 integrin in both primary RGP-derived (WM35) and metastatic melanoma cells (WM9, WM239 and A397 cell lines) [36, 37]. On the other hand, immunohistochemical staining of αvβ3 in human tumor tissue samples did not confirm a positive correlation of integrin expression with the melanoma metastatic phenotype; melanoma *in situ* with a pre-invasive phenotype showed the highest level of αvβ3 expression [38].

Most studies have demonstrated up-regulation of integrin expression in melanoma carcinogenesis; only a few integrin receptors have been found to reduce their expression during disease progression. Ziober et al. [39] found that acquisition of a highly metastatic phenotype by melanoma cells was accompanied by loss of α7β1 expression.

Enhancement of the expression of most integrins promotes conversion of melanoma from the RGP to the VGP and then acquisition of metastatic competence. The switch in expression from LN-binding to FN-binding integrins was shown to contribute to the movement of melanoma cells from the epidermis to the dermis through degraded basement membrane. Apart from induction of αvβ3 expression, the involvement of α3β1 [31], α5β1, and αvβ5 integrins in this process has been found [40].

4. Functions of integrins: role of glycosylation

Integrins participate in a wide range of biological processes, including growth, proliferation, differentiation, survival/apoptosis, and cell-cycle regulation [41–44]. Apart from the adhesion function, they mediate cell signaling events [45–47].

Tumor progression requires comprehensive alteration of normal cell-cell and cell-ECM interactions [34, 48]. Integrins are the main adhesion proteins responsible for these changes, mainly due to their altered expression. They contribute to regulation of such processes as angiogenesis, tumor growth and metastasis, as well as cell proliferation, survival and motility [49–53]. Abundant glycosylation of the extracellular domains of integrins also significantly affects the function of these receptors [2, 54, 55].

Glycosylation is one of the most frequent post-translational modifications of transmembrane and secreted proteins. Both integrin chains are subject to this modification [56]. Integrin α subunits are more profusely N-glycosylated than their β partners. Subunits α3, α5, and αv in the polypeptide sequences contain 13, 14, and 13 potential N-glycosylation sites, respectively, whereas the β1 and β3 chains include 12 and 6 N-glycan-linked sequences, respectively [57]. Intensive glycosylation of integrin chains during post-translational processing results in high content of the sugar component of the whole glycoprotein molecule. Peptide N-glycosidase F (PNGase F) digestion showed that ca. 24 and 25% of the glycoprotein's molecular weight (MW) responds to N-glycans in α3 subunits from WM35 primary and A375 metastatic melanoma cells, respectively. N-oligosaccharides on β1 subunits account for ca. 24 and 33% of total MW in primary and metastatic cells, respectively. In both subunits, the pool of sialic acids increases in metastatic cells in compared with primary melanoma [58] (**Figure 2**). N-oligosaccharides on

the αv integrin subunit from WM793 primary melanoma cells respond to nearly 30% of glycoprotein MW, and from WM1205Lu metastatic cells 28%. Subunit β3 contains 16% of the N-glycans in WM793 cells and 12% of the N-glycans in WM1205Lu cells [59].

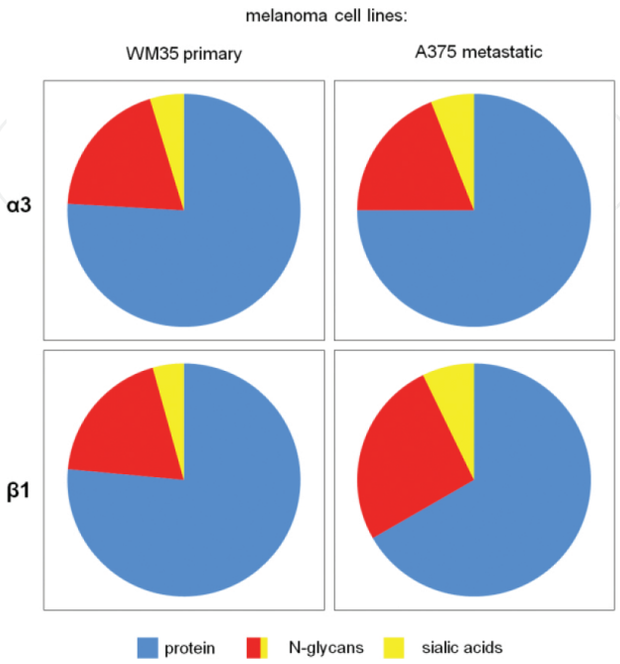

Figure 2. Percentage content of the N-glycan pool and sialic acid in subunits of α3β1 integrin, based on Pocheć et al. [58].

Integrin chains bear all types of N-glycan structures, starting from the evolutionarily oldest structures high-mannose-type, through hybrid glycans, and ending in the most complicated complex-type oligosaccharides [1, 54]. The occurrence of these glycostructures on β1 integrins in B16-F10 melanoma cells depends on the stage of integrin maturation. High-mannose glycans recognized by GNA lectin (*Galanthus nivalis* agglutinin) were abundant on the immature form of β1 integrins with lower molecular weight. The mature, larger β1 chain carried mostly sialylated complex-type structures, identified using DSA (*Datura stramonium* agglutinin) and MAA (*Maackia ammurensis* agglutinin) lectins. Only the completely processed form of β1 integrin was detected at the cell surface of murine melanoma [60].

Glycosylation is crucial to the processing, activation, and functioning of integrins [56, 61]. The function of integrin glycans has been determined mostly using N-glycan synthesis inhibitors, such as castanospermine and N-methyldeoxynojirimycin, which block glucosidases I and II responsible for trimming glucose from the precursor form of N-linked oligosaccharides; 1-deoxymannojirimycin and swainsonine (SW), inhibitors of mannosidase I and II, respectively, and tunicamycin, which abolishes N-glycosylation by inhibiting the action of N-acetylglucosamine-1-phosphotransferase. Other useful tools for assessing N-glycan functioning are recombinant glycosidases, such as PNGase F, which removes glycans N-linked to the protein backbone, and endo-N-acetylglucosaminidase F (Endo F), which cleaves high-mannose and complex-type N-glycans [62].

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Glycosylation of αvβ3 integrin is necessary to assembly of the heterodimer, proteolytic cleavage of the α chain, and cell surface expression of this VN receptor in human melanoma cells. Application of castanospermine and N-methyldeoxynojirimycin decreased αvβ3 surface expression as the result of reduced chain assembly and α polypeptide cleavage. On the other hand, 1-deoxymannojirimycin and SW, inhibitors acting on the later stages of glycan synthesis, did not influence αvβ3 transport to the cell membrane [63]. The importance of N-glycosylation in associating the two subunits was also clearly demonstrated by treating α5βl integrin with Endo F and PNGase F. Enzymatic digestion of purified α5βl integrin resulted in separate precipitation of the α and β polypeptide chains; undigested integrin subunits underwent co-precipitation [64]. Further research using sequential side-directed mutagenesis showed that N-glycosylation of the I-like domain of the βl subunit is essential for the formation of the α5βl heterodimer and for integrin functioning [65].

Cell surface carbohydrates present on adhesion proteins are involved in adhesive and migratory events crucial to each step of the metastatic process. In early studies by Chammas et al., it was found that glycosylation of the β1 subunit complexed with α6 integrin is essential for interaction with LN. Binding of B16-F10 melanoma cells to LN via α6βl integrin was nearly abolished in tunicamycin-treated cells and after treating LN with Endo F/PNGase F [66]. Similarly, digestion of α5βl integrin with a mixture of Endo F and PNGase F led to the loss of FN binding [64]. Lectin analysis showed that both subunits of α6βl integrin bear mainly sialylated complex-type N-glycan structures. Exoglycosidase treatment identified galactose residues on the α subunit as the LN-binding determinants involved in cell adhesion to this ECM ligand. The integrin β chain, abundant in complex-type structures, whose synthesis was inhibited by SW (which blocks the formation of complex-type glycans, among them β1,6-branched glycans), was associated with cell spread but not cell adhesion [67]. Also, human metastatic malignant melanoma cell lines LOX and FEMX treated with tunicamycin showed significantly weaker adhesion to LN and to a lesser extent to type IV collagen. Inhibition of N-glycan synthesis by tunicamycin resulted in reduction of LOX and FEMX invasion through Matrigel-coated chambers, as well as diminution of human melanoma aggregation [68].

5. Alterations of integrin glycosylation in melanoma carcinogenesis

The vast majority of studies on integrin glycosylation in melanoma have used mouse melanoma cell line B16-F10 and phenotypic variants of it that show different degrees of invasive potential, mainly the weakly invasive cell lines B16-F1 or B16-Flr, and B16-BL6 cells selected for their higher ability to metastasize to the lungs [60, 67, 69–73], as well as human melanoma cell lines derived from each stage of melanoma progression, most of which were established by Herlyn's group [3].

It has been demonstrated that the glycosylation profile of integrins depends on the stage of melanoma development [37, 58, 59, 74] and the location of the metastasis [75, 76] and that glycosylation is essential to the interaction between integrin and ECM proteins during adhesion and migration processes [58, 75, 76]. These studies have produced ample evidence

for the presence of glycoforms associated with melanoma carcinogenesis on α3β1, α5β1, and αvβ3 integrins. The changes in the β1,6 branching of complex-type N-glycans, and their sialylation, have been observed on these integrins during human melanoma progression.

5.1. Branched complex-type N-glycans

One of the well-characterized changes in N-glycosylation is enhanced expression of β1,6-N-acetylglucosaminyltransferase V (GnT-V) and its products, N-acetylglucosamine (GlcNAc) β1,6-branched N-linked oligosaccharides, observed in the tumorigenesis of many cancers [77–81], including melanoma [74]. β1,6-branched N-glycans are important in invasion of the basement membrane [82] and acquisition of metastatic competence [83]. β1,6 branching of glycans on integrin chains has been described in studies of mouse and human melanoma.

The presence of β1,6-branched complex-type oligosaccharides on the integrin receptors that bind LN and FN was first shown by Chammas et al. in mouse melanoma cell line B16-F10 [70] and then confirmed on the β1 subunit sharing integrins in this parent cell line and its highly invasive B16-BL6 variant [73, 84, 85]. Significantly enhanced β1,6 branching found on highly invasive B16-BL6 cells resulted in their more efficient invasion and migration, as well as impaired adhesion to different ECM proteins (LN, FN, VN, type I and type IV collagen, hyaluronic acid, and Matrigel). Inhibition of β1,6 branching on two levels—expression of GnT-V by cell transfection (using antisense cDNA), and oligosaccharide synthesis (using SW)—decreased metastasis and invasion of B16-BL6 cells by half, and reduced the formation of metastatic colonies in lungs [73]. Later it was found that α3β1 and α5β1 integrins on mouse B16-BL6 cells carry β1,6-branched oligosaccharides and that β1,6-glycosylation of integrins has an effect on the spread of melanoma cells on FN and Matrigel. Interestingly, β1,6-branched glycans on α3β1 weakened the association of integrin with CD151 tetraspanin [85]. Earlier the crucial role of glycosylation in the interaction of α3β1 with CD151 had been described in work using MDA-MB-231 human breast cancer cells [86]. For B16-BL6 mouse melanoma cells, it was shown (by co-precipitating α3β1 and CD151 from SW-treated cells) that β1,6-branched N-glycans regulate the association of CD151 with this integrin [85].

In human melanoma cells, we demonstrated β1,6 branching of cancer-associated integrin subunits such as α2, α3, α4, α5, αv, β1, and β3 [37]; integrin heterodimers of special importance in melanoma carcinogenesis are α3β1 [58, 75], α5β1 [74], and αvβ3 [59, 75, 87].

A number of studies have confirmed the involvement of α3β1 integrin in melanoma development through its participation in cell adhesion, migration, and invasion [88–90]. The ability of α3β1 to promote melanoma metastasis results from its enhanced synthesis [91, 92] and also from altered glycosylation of it, particularly enhanced β1,6 branching [58, 74, 75].

Glycosylation of α3β1 integrin was first recognized as a factor promoting tumorigenesis in human colon carcinoma cells. Sialylated β1,6-branched Asn-linked oligosaccharides with short poly-N-acetyllactosamine units were found on both integrin subunits. Due to their role in cancer development, they were suggested to be oncodevelopmental carbohydrate epitopes [93].

Different techniques have been employed to analyze α3β1 integrin glycosylation in detail in melanoma cells derived from primary and metastatic tumors. The use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) showed the presence of tetra-antennary complex-type glycans on the β1 subunit in highly metastatic A375 melanoma cells but not in WM35 cells from the primary site. The reaction of affinity-chromatography-purified α3β1 integrin with *Phaseolus vulgaris* agglutinin (PHA-L) revealed that complex-type glycans are β1,6-branched in the α3 subunit from metastatic but not from primary cells [58]. The presence of GlcNAc β1,6-branched glycans on α3β1 in A375 metastatic cells was confirmed by tandem mass spectrometry (MS/MS) of PHA-L-positive glycoproteins eluted in lectin-affinity chromatography [36]. The absence of this type of branching on the α3 subunit in WM35 primary melanoma was thoroughly documented by MS/MS identification of PHA-L bound proteins and two-sided control of integrin glycosylation: immunoblotting in PHA-L-eluted material and PHA-L blotting in immunoprecipitation [37]. In two other metastatic melanoma cell lines (WM9 and WM239), β1,6 branching of α3β1 integrin was shown using MS/MS identification of PHA-L-bound glycoproteins [37] and confirmed using MALDI-MS and PHA-L precipitation [75]. The amount of glycans with β1,6-linked antenna increased in WM1205Lu metastatic melanoma as compared to WM793 primary cells [74]. Using normal-phase high-performance liquid chromatography (NP-HPLC), however, Link-Lenczowski et al. [94] did not observe differences in α3β1 glycosylation profiles between WM115 primary and WM266-4 metastatic human melanoma cell lines originating from the same patient.

The role of α5 integrin in promoting melanoma metastasis has been shown in uveal [95] and cutaneous melanoma [29, 96]. An increase of the metastatic potential of melanoma is accompanied by enhancement of α5 integrin expression [30, 97]. In highly metastatic B16-F10 melanoma cells, the level of α5 integrin was conspicuously elevated as compared to weakly metastatic B16-F1 cells. Pulmonary metastasis in mice as well as the adhesion and spread of B16-F10 cells to FN *in vitro* was significantly reduced after blocking of α5 integrin by a specific antibody. The loss of α5-mediated melanoma cell-FN anchoring promoted apoptosis of B16-F10 cells [30].

Integrin α5β1 is also a carrier of β1,6-branched glycans in metastatic cells, but on the α5 subunit from primary melanoma, this type of branching was not detected. In each of three analyzed metastatic cell lines (WM9, WM239 and A375), the α5 subunit oligosaccharides were β1,6-branched [37], but not the α5 chain in WM35 melanoma cells [36], as determined using MS/MS analysis of PHA-L-positive glycoproteins. A comparison of α5 integrin chains from early VGP and metastatic lesion cells showed an uptrend of β1,6 branching during acquisition of metastatic competence [74]. These findings suggest that GlcNAc β1,6-branched structures appear earlier in melanoma development on the β1 subunit than on the α3 and α5 chains and that in melanoma cancerogenesis their content is more stable on the β1 subunit than on the α3 and α5 chains [36, 37, 74].

Glycosylation of integrin αvβ3 is still rather poorly understood [54], although it is well known that this integrin is associated with the metastatic potential of melanoma [33, 35, 98]. Our studies using two genetically related melanoma cell lines showed the presence of β1,6-branched complex-type structures on primary and metastatic cells, but we did not observe

differences in the β1,6 branching of αvβ3 glycans during the transition from primary VGP melanoma to its metastatic variant. PHA-L precipitation and SW treatment gave similar levels of β1,6 branching in both αvβ3 subunits in cell lines WM793 and WM1205Lu [59]. This type of glycan was also present on the αv subunit from RGP-derived WM35 melanoma cells, but β1,6 branching was not found on the β3 chain from these cells [36]. Integrin αvβ3 from three metastatic cell lines (WM9, WM239 and A375) of varying origin showed expression of these structures [37, 75].

The phenomenon of competition for a substrate between N-acetylglucosaminyltransferase III (GnT-III) and GnT-V is well documented in N-glycan biology. GnT-III activity during N-glycan processing can suppress the biological functions of GnT-V; it results in reduction of N-glycan β1,6 branching. With respect to integrins, this was first shown on α3β1 in human gastric cancer cell line MKN45 [99]. In B16 melanoma cells, ectopic expression of GnT-III was shown to retard cell metastasis through inhibition of GnT-V activity: the absence of GnT-V products was associated with attenuation of malignant cell motility [83]. Our group showed a significant decrease of bisecting GlcNAc content on αvβ3 integrin subunits during the transition from the VGP to the metastatic stage, but it was not associated with any change in the amount of β1,6-branched glycans on this integrin [59], although previously in this pair of related cell lines (WM115 vs. WM1205Lu), we observed significant upregulation of GnT-V expression [74].

Integrin-mediated cell migration requires adhesion of cells to ECM substrates and is essential for dissemination of the tumor to distant organs during metastasis [100], so the role of integrin glycosylation is frequently assessed in different adhesion and migration tests. Functional studies have clearly shown that β1,6 branching on cell surface adhesion receptors, mainly integrins, promotes melanoma cell migration [101], and invasion [90].

The contribution of α3β1 integrin's N-glycans to its binding with its ECM ligands was demonstrated using affinity-chromatography-purified integrin from WM35 primary and A375 metastatic melanoma cells. In direct ligand-binding assays, de-N-glycosylated α3β1 integrin showed enhanced binding of both melanoma cell lines to LN, type IV collagen and FN, except for the binding of α3β1 from WM35 to FN [58]. Enzymatic removal of N-glycans from this integrin in two metastatic melanoma cell lines from metastases of different origin (WM9 and WM239) also resulted in enhanced binding of α3β1 to LN5 [75].

Of the ECM proteins, fibronectin is the major α5β1 ligand [102] and therefore is the one most frequently chosen for assays evaluating the involvement of α5β1 integrin in adhesion and migration processes. β1,6 branching of FN receptors was shown to contribute to migration of metastatic melanoma on FN, but not to primary cell migration [74].

N-glycan-dependent binding of integrins to the ECM triggers intracellular pathways via phosphorylation of cytoplasmic kinases. FAK is one of the first proteins recruited to integrins aggregated within the cell membrane. Activation of signal pathways leads to the expression of different genes that control cell growth, differentiation, tumor invasion and metastasis [103, 104]. Changes in integrin glycosylation affect intracellular signals triggered by melanoma cell binding to the ECM. Dual immunostaining of melanoma cells growing on VN showed co-localization of αvβ3 integrin and FAK, a downstream target of integrins, in focal adhesion sites

of melanoma cells. Overexpression of GnT-V in human WM266-4 metastatic melanoma cells up-regulated αvβ3-integrin-mediated FAK phosphorylation and cell migration on VN, while inhibition of β1,6 branching by SW-treatment reduced FAK signaling activation in both A375 and WM266-4 metastatic cells [87].

An interesting aspect of integrin glycans' involvement in melanoma metastasis is their participation in ECM degradation through regulation of the activity of matrix proteases, such as urokinase-type plasminogen activator (uPA) and metalloproteinases (MMPs). Integrins interact with urokinase-type plasminogen activator receptors (uPARs) in the cell membrane [105]. A urokinase-type plasminogen activator (uPA), acting via its receptor (uPAR), catalyzes the activation of plasmin from plasminogen, and the plasmin initiates a proteolytic cascade leading to degradation of the ECM [106, 107]. Our work demonstrated that β1,6-branched oligosaccharides on αvβ3 and α3β1 integrins are essential for the association of the uPAR with integrins in human melanoma cell lines WM9 and WM239, seen in the failure of co-precipitation of the two integrins with the uPAR in SW-treated cells. Adhesion of the two melanoma cells to VN was dependant on β1,6 branching of αvβ3 and α3β1 integrins in a cell-line-specific manner [76].

N-glycans with β1,6-branched antennae on melanoma integrins also modify the activity of metalloproteinases (MMPs). PHA-L precipitation revealed that β1 integrins from B16-BL6 cells are more β1,6-branched than the parent cells with lower invasion ability. β1,6-glycosylation of β1 integrin receptors affected the activation of membrane-tethered forms of metalloproteinases (MT1-MMPs). The association of β1,6-glycosylation-suppressed β1 integrin with MT1-MMPs was more severely affected in B16-BL6 cells than in the parent cells, suggesting that integrin β1,6 branching contributes to melanoma invasion also through activation of MMPs [84].

5.2. Sialylation

Sialic acid-linked α2,3 or α2,6, mostly in terminal positions of the oligosaccharide, gives these molecules a negative charge [108, 109] that significantly influences cell interaction mediated by sialylated adhesion proteins, among them integrins [110]. Hypersialylation of cell surface receptors is important in tumor invasion and metastasis [111]. MAA is the lectin commonly used to analyze a pool of α2,3-linked sialic acid, while a lectin from *Sambucus nigra* (SNA) is specific for α2,6-linked sialic acid [112]. The presence of sialic acids on α3β1, α5β1, and αvβ3 integrins in melanoma cells was confirmed in each stage of melanoma progression [58, 59, 75].

One of the first studies on integrin sialylation in melanoma employed mouse melanoma cell lines differing in their metastatic ability. Analysis of melanoma cell sialylation using HPLC and digestion by *Vibrio cholerae* sialidase did not show changes in the total content of cell surface sialic acids on mouse B16 metastatic melanoma cell variants differing in their invasive potential [69]. Research on specific adhesion proteins provided more detailed information. Integrin β1 from both B16-F1 mouse metastatic melanoma and its weakly metastatic wheat germ agglutinin-resistant mutant Wa4-b1 was found to contain high-mannose and bi-, tri-, and tetra-antennary complex-type N-oligosaccharides. Sialylation of the β1 subunit was significantly decreased in mutant melanoma cells with low metastatic ability. Alteration of β1 integrin

glycosylation resulted in reduction of the mutant's metastatic potential and adhesion to FN and LN, as compared to the parent cells [71]. Higher β1,6 branching of complex-type glycans on more invasive B16-BL6 melanoma cells versus the parent B16-F10 line was correlated with an increase of α2,3-linked and α2,6-linked sialic acid content as determined using MAA and SNA staining in flow cytometry. Hypersialylation of B16-BL6 cells resulted in their higher motility and stronger adhesion to selected ECM proteins [73]. Further results for this pair of murine melanoma cell lines were obtained by lectin blotting: α2,6-linked sialic acid especially increased on B16-BL6 glycans as a result of enhanced β1,6 branching. α2,6-desialylation and down-regulation of the sialyltransferase ST6Gal-I, which transfers sialic acids to oligosaccharides and catalyzes the formation of α2,6 linkage, negatively affected adhesion and invasion of B16-BL6 cells [113]. In turn, a study by Chang and colleagues showed that α2,3-linked sialic acid is important in the metastasis of B16-F10 cells. Soyasaponin I (Ssa I), which specifically inhibits the expression of α2,3-linked sialic acids, reduced the migratory ability of melanoma, up-regulated cell adhesion to ECM proteins, and impaired pulmonary metastasis [114].

Our studies using different human melanoma cell line models indicated reduction of α2,3 sialylation on the α3 integrin subunit, and of α2,6 sialylation on αvβ3 integrin, in melanoma progression [74, 59]. Lectin-probed Western blotting showed that the β1 subunit from both cell lines and the α3 subunit from primary melanoma cell line WM35 had both types of sialic acid linkage, while the α3 subunit from metastatic cell line A375 lost its α2,3 glycosidic linkage [58]. Using genetically matched cell lines WM793 and WM1205Lu from the last two stages of melanoma progression, we observed a shift in the sialylation of αvβ3 integrin during the transition from VGP to metastatic tumor. Lectin MAA and SNA precipitation as well as digestion by two neuraminidases with narrower (α2,3) and wider (α2-3,6,8) specificity showed that α2,6-linked sialic acid was reduced, whereas α2,3-linked sialic acid increased on both integrin subunits from metastatic lesion cells. In a wound-healing assay, migration of melanoma cells on VN in the presence of both lectins was affected only in the metastatic cell line [59]. Lectin flow cytometry of another pair of related melanoma cell lines (WM115 derived from RGP/VGP vs. WM266-4 from lymph node metastasis) indicated a more than fourfold increase of cell surface α2,3 sialylation during the acquisition of metastatic competence. Despite these differences in surface α2,3 sialylation, the reduction of migration by MAA-treated primary and melanoma cells was comparable, suggesting the involvement of receptor(s) other than αvβ3 integrin and its/their sialylation in metastatic cell migration (data not published).

Digestion of α3β1 glycans with a broad-specificity neuraminidase from *Arthrobacter ureafaciens* led to stronger binding of the integrin to various ECM components (LN, FN, and type IV collagen) in both primary and metastatic melanoma cells. Interestingly, removal of the sialic acids by neuraminidase enhanced integrin binding significantly more than complete de-N-glycosylation did, suggesting an important role of desialylated N-oligosaccharides in integrin-ECM interactions [58]. For efficient cell-ECM adhesion, protein-protein interactions apparently are not enough, and glycosylation is needed to regulate this binding.

Attachment of α2,8 to underlying glycans by sialic acid is rather rarely detected on integrins. A study using human melanoma cell line G361 is one of the few that have demonstrated the

presence of α2,8-bound sialic acid on α5β1 integrin—and the role of this type of sialylation in FN binding. Desialylation using an enzyme from *Arthrobacter ureafaciens* specific for α2-3,6,8-linked sialic acids resulted in reduction of α5β1-mediated adhesion to FN, an effect not observed for neuraminidase, which cleaves only α2-3,6 linkages [115].

Undoubtedly, the sialylation state of integrins contributes to the metastatic potential of mouse and human melanoma, but there are blank spots in our understanding of the role of α2,3-linked and α2,6-linked sialic acid in melanoma progression. Further studies should establish precisely how sialylation becomes altered, and its contribution to the disease phenotype.

6. Conclusions

The search for glyco-biomarkers on integrins in melanoma progression motivates a host of studies performed by different research groups. Identification of universally present alterations of glycans on adhesion molecules, among them integrins—and elucidation of the molecular mechanisms of these changes—will boost our understanding of how melanoma cells acquire the ability to escape the primary tumor and spread through the body. Enhanced β1,6 branching and altered sialylation are the main glyco-features of integrin glycosylation in melanoma progression. The functional consequences of surface glycosylation rearrangements in melanoma progression must be known if we are to find effective ways to stop the process of carcinogenesis. The vast majority of studies on integrin glycosylation in melanoma cells have used cells cultured *in vitro*. A hugely important task for future research is to verify the results obtained from *in vitro* studies of tumor tissue from patients with melanoma, so that those findings can be applied for prevention and treatment of melanoma.

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